

## Metabolic Initiation of Differentiation and Secondary Metabolism by *Streptomyces griseus*: Significance of the Stringent Response (ppGpp) and GTP Content in Relation to A Factor

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Received 28 January 1987/Accepted 30 April 1987

I investigated the significance of the intracellular accumulation of guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and of the coordinated decrease in the GTP pool for initiating morphological and physiological differentiation of *Streptomyces griseus*, a streptomycin-producing strain. In solid cultures, aerial mycelium formation was severely suppressed by the presence of excess nutrients. However, decoyinine, a specific inhibitor of GMP synthetase, enabled the cells to develop aerial mycelia in the suppressed cultures at concentrations which only partially inhibited growth. A factor (2S-isocapryloyl-3S-hydroxymethyl- $\gamma$ -butyrolactone) added exogenously had no such effect. Decoyinine was also effective in initiating the formation of submerged spores in liquid culture. The ability to produce streptomycin did not increase but decreased drastically on the addition of decoyinine. This sharp decrease in streptomycin production was accompanied by a decrease in intracellular accumulation of ppGpp. A relaxed (*rel*) mutant was found among 25 thiopeptin-resistant isolates which developed spontaneously. The *rel* mutant had a severely reduced ability to accumulate ppGpp during a nutritional shift-down and also during postexponential growth and showed a less extensive decrease in the GTP pool than that in the *rel*<sup>+</sup> parental strain. The *rel* mutant failed to induce the enzymes amidinotransferase and streptomycin kinase, which are essential for the biosynthesis of streptomycin. The abilities to form aerial mycelia and submerged spores were still retained, but the amounts were less, and for both the onset of development was markedly delayed. The decreased ability to produce submerged spores was largely restored by the addition of decoyinine. This was accompanied by an extensive GTP pool decrease. The *rel* mutant produced A factor normally, indicating that synthesis of A factor is controlled neither by ppGpp nor by GTP. Conversely, a mutant defective in A-factor synthesis accumulated as much ppGpp as did the parental strain. It was concluded that morphological differentiation of *S. griseus* results from a decrease in the pool of GTP, whereas physiological differentiation results from a more direct function of the *rel* gene product (ppGpp). It is also suggested that A factor may render the cell sensitive to receive and respond to the specified signal molecules, presumably ppGpp (for physiological differentiation) or GTP (for morphological differentiation).

In bacteria, differentiation and induction of secondary metabolism start concomitantly in response to nutrient limitation. The study of differentiation in *Streptomyces* spp. has been especially of interest because this genus produces numerous antibiotics by secondary metabolism. The coupling of antibiotic production to morphological differentiation suggests a mechanistic connection which has not been demonstrated. The two processes do, however, often occur in parallel, (5). Typically, *Streptomyces* spp. develop aerial mycelia from substrate mycelia prior to the formation of spores (aerial spores) when cultured on a suitable solid medium. Some *Streptomyces* spp., including *Streptomyces griseus*, are able to form spores even in liquid culture (called submerged spores) without forming aerial mycelia if the cells are exposed to appropriate nutritional conditions (16, 36). Another distinguishing characteristic is the fact that many *Streptomyces* spp. produce endogenous signal molecules called autoregulators (18, 29). The best studied is A factor (2S-isocapryloyl-3S-hydroxymethyl- $\gamma$ -butyrolactone), which is essential for the production of both streptomycin and aerial mycelia by *S. griseus* (11, 15, 17, 19). Mutants defective in A-factor synthesis lose the capability of producing both streptomycin and aerial mycelia. Exogenous addition of A factor restores both phenotypes. Such a simulta-

neous restoration is also known in a *Streptomyces griseoflavus* mutant defective in arginine synthesis, whose differentiation and antibiotic production can be restored by adding citrulline (28). Of interest is the fact that a well-sporulating *S. griseus* mutant accumulates a proteinlike substance, designated factor C, in liquid cultures which restores the capability of sporulation of a certain *S. griseus* mutant (3). Thus, the genus *Streptomyces* provides a feasible system for studying the initiation of differentiation and secondary metabolism.

The stringent response and the involvement of guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) have been studied extensively in *Escherichia coli* (4, 7). Other procaryotes may be different (2, 6, 31). By isolating and analyzing the relaxed mutants of *Streptomyces* spp., I have stressed the significance of the stringent response for the initiation of both differentiation and secondary metabolism of this genus. It was found that aerial mycelium formation results from a decrease in the pool of GTP (e.g., caused by the stringent response), whereas antibiotic production results from a different effect of the stringent response (23–25). This notion was consistent with what obtained earlier with *Bacillus subtilis* (27). Working with *S. griseus* IFO 13189, an A-factor-producing strain, I report here a significant contribu-

tion of the stringent response and of the coordinated decrease in GTP for initiating differentiation and secondary metabolism, clarifying the relationship with the role of A factor.

## MATERIALS AND METHODS

**Source of drugs.** ppGpp and pppGpp were purchased from Sanraku Co., Fujisawa, Japan, and Casamino Acids (vitamin free) were from Difco Laboratories, Detroit, Mich. [2-<sup>14</sup>C]uracil was from New England Nuclear Corp., Boston, Mass. Decoyinine and A factor were donated by J. E. Grady, The Upjohn Co., Kalamazoo, Mich., and T. Beppu, University of Tokyo, Japan, respectively. Thiopeptin was a commercial product of our company. All medium components were from commercial sources.

**Strains and preparation of mutants.** *S. griseus* IFO 13189, a wild-type, prototrophic streptomycin-producing strain, was provided by the Institute of Fermentation, Osaka, Japan. The spontaneous thiopeptin-resistant mutants were obtained as resistant colonies, which developed with a frequency of about  $3 \times 10^{-7}$  20 days after spores were spread on sporulation agar (see below) supplemented with 0.5% Polypeptone (BBL Microbiology Systems, Cockeysville, Md.) plus 3  $\mu$ g of thiopeptin per ml. (Growth of the parental strain was completely suppressed with 2  $\mu$ g of thiopeptin per ml.) The resistant clones were purified by single-colony isolation. Mutants defective in A-factor synthesis were found at a high incidence among cells which had grown at a high temperature (36°C) for 24 h, as described by Hara and Beppu (11). One of them, designated 14, was used in this study. Strains were preserved at 4°C after cultivation on sporulation agar at 30°C for 1 week. Mutant strains were cloned every month for subsequent storage. This procedure was especially necessary for the *rel* mutant, because the *rel* mutant was found to be unstable genetically, with highly frequent changes in colony morphology during storage.

**Media.** Synthetic medium was as follows (per liter): glucose, 20 g; glycine, 5 g; sodium citrate (dibasic), 10 g; MgSO<sub>4</sub> (dehydrated), 10 g; NaCl, 5 g; KH<sub>2</sub>PO<sub>4</sub>, 0.5 g; CaCl<sub>2</sub>, 50 mg; ZnSO<sub>4</sub> · 5H<sub>2</sub>O, 30 mg; FeSO<sub>4</sub> · 5H<sub>2</sub>O, 25 mg; CuSO<sub>4</sub> · 5H<sub>2</sub>O, 16 mg; and MnSO<sub>4</sub> · 4H<sub>2</sub>O, 12 mg (adjusted to pH 7.3 with NaOH). A large amount of magnesium sulfate was included in the medium because it greatly stimulated streptomycin production. In some experiments, magnesium sulfate was reduced to 1 g/liter. The medium was sterilized by autoclaving at 120°C for 20 min apart from the glucose solutions, which were autoclaved separately. SPY medium was as follows (per liter): soluble starch, 30 g; Polypeptone, 30 g; yeast extract, 2 g; MgSO<sub>4</sub> (dehydrated), 10 g; CaCl<sub>2</sub>, 50 mg; ZnSO<sub>4</sub> · 5H<sub>2</sub>O, 30 mg; FeSO<sub>4</sub> · 5H<sub>2</sub>O, 25 mg; CuSO<sub>4</sub> · 5H<sub>2</sub>O, 16 mg; and MnSO<sub>4</sub> · 4H<sub>2</sub>O, 12 mg (adjusted to pH 7.2 with NaOH). GYM medium was as follows (per liter): glucose, 4 g; yeast extract, 4 g; malt extract, 10 g; peptone (Nz-amine, type A; Wako Chemicals), 1 g; NaCl, 2 g; and 1 M MOPS (morpholinopropanesulfonic acid; pH 7.2 by KOH), 50 ml. Sporulation agar was as follows (per liter): soluble starch, 20 g; yeast extract, 4 g; and agar, 20 g (adjusted to pH 7.0 with NaOH). Plates (diameter, 8.5 cm) consisted of 25 ml of medium. Sporulation medium (liquid medium for submerged spore formation) contained (per liter) NaCl (5 g), KCl (0.5 g), MgSO<sub>4</sub> (0.25 g), ZnSO<sub>4</sub> · 7H<sub>2</sub>O (10 mg) in addition to the above components (without agar). The salts described above were essential to produce abundant submerged spores.

**Growth conditions.** All the cultivations, including plate cultures, were carried out at 30°C. A spore suspension for

inoculation was prepared by scraping the spores from a slant of sporulation agar with an inoculating loop and transferring the spores to a sterile test tube containing approximately 100 glass beads (diameter, 3 mm) and 5 ml of saline (0.9% NaCl). After continuous blending on a vortex mixer for 2 min (to avoid clumping during growth), the monospore suspension (4 ml; ca.  $8 \times 10^8$  spores) was inoculated into SPY medium (50 ml in a 250-ml flask). After 2 days of cultivation on a rotary shaker (230 rpm), cells were harvested by centrifugation ( $8,000 \times g$ , 10 min), washed twice with 100 ml of saline, and suspended in the original volume of saline. Washed cells (2 ml) were inoculated into 50 ml of synthetic medium or synthetic medium supplemented with 1% (wt/vol) vitamin-free Casamino Acids (in a 250-ml flask) and then cultured on a rotary shaker. In some experiments, cultured broth (1 ml) was inoculated into fresh SPY medium (50 ml) without cell washing and then cultured with rotary shaking.

To study the formation of submerged spores, 2 ml of the monospore suspension described above was directly inoculated into sporulation medium (50 ml in a 250-ml flask) and cultured on a rotary shaker for 48 h.

**Assay of nucleotide pools.** The intracellular concentrations of nucleotides, including ppGpp and pppGpp, were assayed by high-performance liquid chromatography (HPLC) after extraction with 1 N formic acid as previously described (24). Determination of dry cell weight was also described (24). A high-ionic-strength buffer (0.5 M KH<sub>2</sub>PO<sub>4</sub> plus 0.5 M Na<sub>2</sub>SO<sub>4</sub>) was adjusted to pH 5.4 instead of 5.0 (used in the original study [26]), because separation of GTP from ATP was much improved. To normalize the amounts of nucleotides found in different cultures, the amounts were expressed relative to the dry cell weight measured at the time of harvesting, i.e., picomoles per milligram (dry weight) of cells.

**Assay for streptomycin and A factor.** Streptomycin was determined by a disk-plate method with *B. subtilis* ATCC 6633 as a test organism. The assay plate (diameter, 8.7 cm) contained 10 ml of Mueller-Hinton medium plus 0.8% agar.

A factor was assayed by using an *S. griseus* mutant (number 14) defective in A-factor synthesis. A factor produced in a medium was always extracted with an equal volume of chloroform, and the extract was then assayed by the method of Hara and Beppu (11) with slight modification. In some experiments, A factor was roughly assayed by measuring the zones of aerial mycelia produced around the disks containing A factor, as follows. The mycelia of *S. griseus* mutant 14 were spread on sporulation agar (10 ml per 8.5-cm-diameter plate), and paper disks soaked with 40  $\mu$ l of chloroform extracts or standard A factor solutions were put on the plates. These plates were then incubated at 30°C for 48 h. Paper disks containing 0.05 and 0.1  $\mu$ g of A factor produced zones of aerial mycelia 15 and 20 mm in diameter, respectively.

**Enzyme assays.** Crude enzyme preparation was obtained by a sonic disruption (ca. 2 min) of cells suspended in 50 mM phosphate buffer (pH 7.4) for the amidinotransferase assay, 10 mM Tris hydrochloride buffer containing 10 mM MgCl<sub>2</sub>, 25 mM NH<sub>4</sub>Cl, and 0.6 mM mercaptoethanol (pH 7.8) for the streptomycin kinase assay, or 50 mM Tris hydrochloride buffer (pH 7.2) for the NADP-glycohydrolase assay. They were centrifuged at  $15,000 \times g$  at 3°C for 20 min. The supernatant was used as a crude enzyme preparation. It contained 10 to 20 mg of protein per ml.

Amidinotransferase was determined as described by Walker (38) by measuring transamidination from L-arginine to hydroxylamine, which yields hydroxyguanidine. One unit

of amidinotransferase was defined as the amount that catalyzes the formation of 1  $\mu$ mol of hydroxyguanidine per h at 37°C.

Streptomycin kinase was assayed as described by Hara and Beppu (12) by measuring the decrease in biological activity of streptomycin. Before the assay, the crude enzyme preparation was diluted suitably (e.g., 1/4, 1/20) with the buffer described above. The reaction was carried out at 30°C for 1 h and then stopped by a heat treatment at 100°C for 30 s. One unit of streptomycin kinase was defined as the amount that catalyzes the formation of 1  $\mu$ mol of inactivated streptomycin per h.

NADP-glycohydrolase was assayed by the method of Voronina et al. (37). The reaction was carried out at 37°C for 10 min and was stopped by soaking in an ice-cold water bath. One unit of NADP-glycohydrolase was expressed as the amount that catalyzes the decrease of 1 nmol of NADP per min.

Protein was measured by the method of Lowry et al. (21) with serum albumin as the standard.

**Streptomycin resistance.** Cells of each strain, grown in SPY medium for 2 days, were inoculated at a high dilution rate (1:200) into the fresh SPY medium containing various amounts of streptomycin. Growth was observed after 2 days of incubation at 30°C with shaking.

**Determination of RNA synthesis.** RNA synthesis was determined by measuring the incorporation of [2-<sup>14</sup>C]uracil into an acid-precipitable portion as previously described (24).

**Assay of submerged spores.** Before the spore titers were determined, cultured broth was treated with sonication to disperse spores. Vegetative mycelia were broken down largely by this treatment. After suitable dilution, spore titers were determined by direct counting of spores in a Petroff-Hauser counting chamber with a Nikon type 104 microscope with phase-contrast optics.

**Reproducibility.** The experiments shown in each figure or table were repeated two to four times to confirm reproducibility (except for that shown in Fig. 2, which was not repeated); representative results are presented.

## RESULTS

**Initiation of aerial mycelium formation.** *S. griseus* 13189 developed aerial mycelia from substrate mycelia 2 days after inoculation when incubated on nutritionally poor solid medium, i.e., sporulation agar. Aerial mycelium formation, however, was severely suppressed during 8 days of incubation by supplementation with 2% (wt/vol) Casamino Acids (Fig. 1). This suppression was not due to pH, which remained at 7.0 to 7.2 during incubation. The replacement of soluble starch with 1 to 3% (wt/vol) glucose did not suppress the development of aerial mycelia; instead, the appearance of aerial mycelia was prompted by its replacement. Thus, aerial mycelium formation by *S. griseus* seemed to be subject to nitrogen (but not glucose) suppression.

Under such a suppressible condition, the addition of decoyinine, a specific inhibitor of GMP synthetase, at concentrations which only partially inhibited growth enabled the cells to develop aerial mycelia (Fig. 1). Decoyinine exerted its effect at concentrations higher than 0.1 mM, and the maximal effect was observed at 0.3 to 0.5 mM. Larger amounts of decoyinine severely inhibited growth, and aerial mycelia did not develop. As examined in liquid culture (see below), decoyinine addition resulted in a decrease in the intracellular GTP content. By contrast, A factor did not have such a derepressible effect. A factor (0.001 to 2  $\mu$ g/ml) added

at the time of inoculation did not initiate the development of aerial mycelia on sporulation agar supplemented with 2% Casamino Acids, indicating that the presence of A factor alone was not sufficient for release from the suppression.

**Effect of decoyinine on streptomycin and A-factor production.** The addition of decoyinine did not result in an increase in the ability to produce streptomycin but rather reduced it. Decoyinine virtually had no effect on producing A factor (Fig. 1).

To study further the effect of decoyinine, experiments were carried out in liquid culture with SPY medium, a suitable medium for the production of both streptomycin and A factor. *S. griseus* 13189 produced no streptomycin during exponential growth but began producing it after the end of exponential growth, concomitant with an increase in intracellular ppGpp (60 pmol/mg [dry weight] at 20 h). Streptomycin in the medium reached 90 to 100  $\mu$ g/ml after 48 h of cultivation. In contrast, A factor was produced in parallel with the cell growth, showing a maximal titer of 1.2  $\mu$ g/ml at 28 h, and then it decreased rapidly. When cells were grown in synthetic medium, relatively high ppGpp accumulation (45 to 55 pmol/mg [dry weight]) was detected throughout exponential growth and streptomycin was produced in parallel with the cell growth. If the synthetic medium was supplemented with 2% Casamino Acids, ppGpp accumulation during exponential growth was low (10 to 22 pmol/mg [dry weight]) and streptomycin was produced only after the end of growth.

Similar to agar plate cultures, the addition of decoyinine to the cells growing in SPY medium did not stimulate streptomycin production at any concentrations tested (0.01 to 1 mM) but severely reduced it at concentrations higher than 0.2 mM. The ability to produce A factor was virtually unaffected. The pool size of GTP decreased immediately upon the addition of 0.3 mM decoyinine, without increase in ppGpp (3 to 5 pmol/mg [dry weight] throughout 4 h of incubation) (Fig. 2). These results, together with those for agar plate cultures, indicate that the decrease in GTP content did not result in an initiation of streptomycin production or A-factor production. The observed suppression of streptomycin production upon the addition of decoyinine was not accompanied by the accumulation of ppGpp; the ppGpp content of the cells growing with 0.3 mM decoyinine was only 4 pmol/mg (dry weight) even after 20 h of cultivation. Conceivably, with a relatively severe growth inhibition (by 45%) nutrient(s) were not exhausted, and thus the accumulation of ppGpp was blocked. This possibility was pursued by experiments with a *rel* mutant.

**Isolation of relaxed mutant.** In *B. subtilis*, relaxed (*relC*) mutants, lacking ribosomal protein BS-L11, have been previously recovered as thiostrepton-resistant isolates (33, 34). In the present study, thiopeptin, an analog of thiostrepton (14), was used to isolate drug-resistant mutants of *S. griseus*. Among thiopeptin-resistant isolates, 25 strains selected randomly were tested for their ability to accumulate ppGpp during nutritional shift down, i.e., Casamino Acids deprivation. A strain, designated 3-3, produced severely reduced amounts of ppGpp (about 10-fold less) after shift down, while the other 24 strains produced as much ppGpp as did the parental strain 13189 (Fig. 3). RNA synthesis by the parental strain was markedly reduced upon the deprivation of Casamino Acids, whereas mutant 3-3 continued RNA synthesis for 20 min at a rate higher than that observed in the presence of Casamino Acids (Fig. 4). Thus, mutant 3-3 exhibited the relaxed phenotype with respect to RNA synthesis. The relaxed mutant thus obtained grew normally in

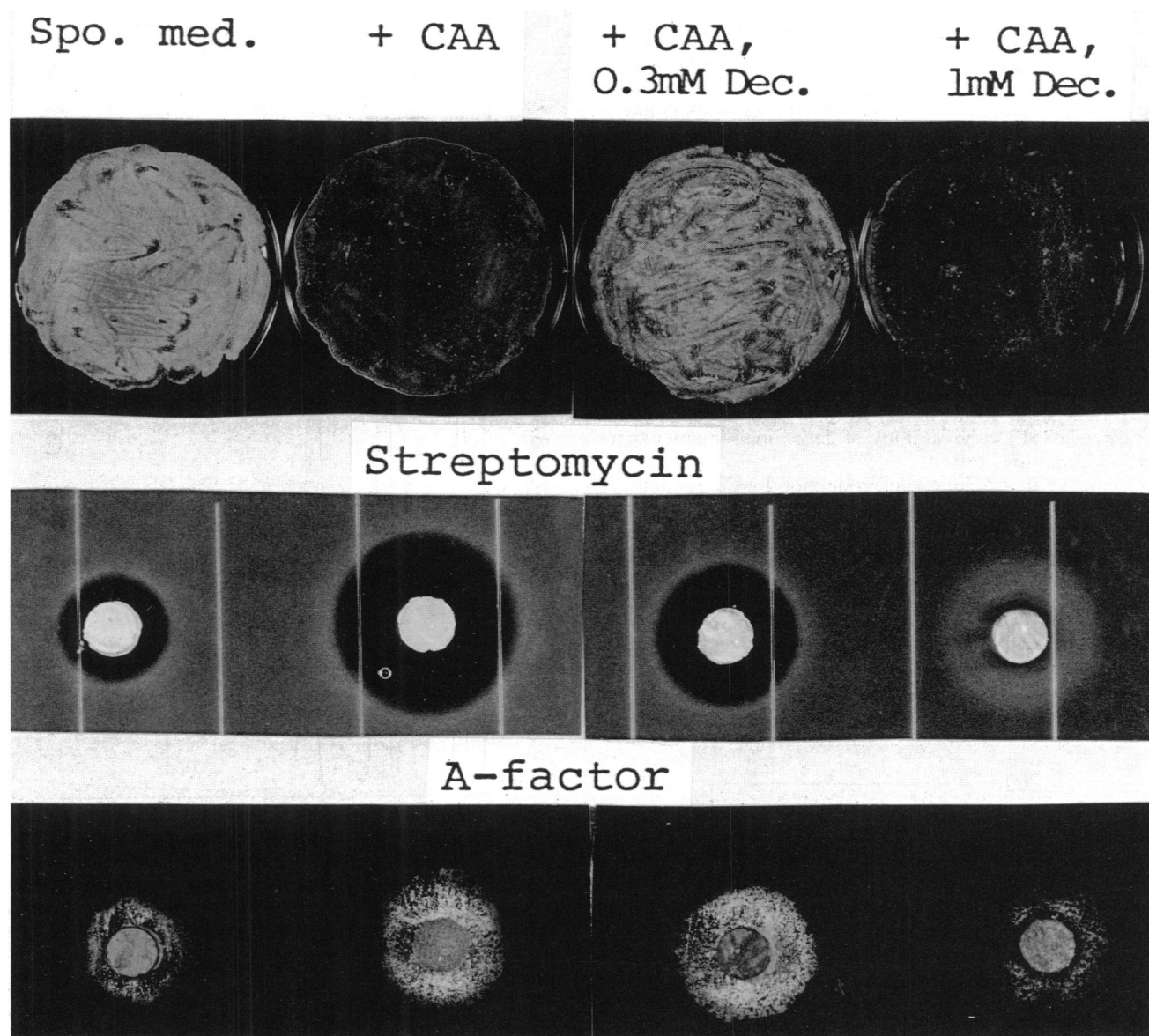


FIG. 1. Development of aerial mycelia by *S. griseus* 13189 on the addition of decoyinine and production of streptomycin and A factor. Spores were spread on sporulation agar (Spo. med.) or sporulation agar supplemented with 2% Casamino Acids (CAA) plus or minus decoyinine (Dec.). The plates were incubated at 30°C for 4 days (upper panel). Agar pieces (diameter, 8 mm) cut from each of the plates after incubation were put in the same order on the assay plate inoculated with *B. subtilis* for the streptomycin assay or on the plate inoculated with *S. griseus* defective in A-factor synthesis for the A-factor assay (lower panel). The plates were incubated at 30°C for 20 h for the streptomycin assay (center panel) or for 2 days for the A-factor assay (lower panel).

synthetic medium and was resistant to thiopeptin up to 7  $\mu\text{g/ml}$ , as determined after 7 days of incubation on agar plates. (The resistance of the parental strain was up to 1  $\mu\text{g/ml}$ .) Mutant 3-3 may be tentatively classified as a *relC* mutant, in analogy with the mutants of *E. coli* and *B. subtilis*, which lack ribosomal protein L-11 (33, 34).

**Characterization of mutants.** The *rel* mutant was strikingly deficient in producing streptomycin in both liquid and agar plate cultures. The parental strain produced 90 to 100  $\mu\text{g}$  of streptomycin per ml in SPY medium, while the *rel* mutant produced only 3  $\mu\text{g/ml}$ . Deficiency in streptomycin production was confirmed with 15 different media which varied in carbon or nitrogen sources. The thiopeptin-resistant mutants which did produce as much ppGpp as the parental strain did

also produced amounts of streptomycin comparable to that produced by the parental strain, indicating the correlation of streptomycin-producing ability with the ability to accumulate ppGpp. The decreased ability of the *rel* mutant to produce streptomycin could not be attributed to an abnormal accumulation of acidic or alkaline metabolites, as the final pH values of the culture media of the parent and mutant strains were similar. In addition, streptomycin added exogenously to the cultures of the *rel* mutant did not decrease, eliminating the possibility of an increased ability to inactivate streptomycin. Resistance of the *rel* mutant to streptomycin (self-resistance) was reduced by a factor of 2 (400  $\mu\text{g/ml}$  and 1 mg/ml for 50% growth inhibition of the *rel* mutant and parental strain, respectively).

The *rel* mutant produced A factor normally in both liquid and agar plate cultures; both the parental and mutant strains produced 1.2  $\mu\text{g}$  of A factor per ml in SPY medium after 20 to 30 h of cultivation. Although the *rel* mutant still retained the ability to form aerial mycelia and spores when cultured on sporulation agar, the amount of aerial mycelia was less than that produced by the parental strain and the onset of aerial mycelium formation was delayed by 2 to 3 days.

Mutant 14, obtained after growth at high temperature (see Materials and Methods), lacked the ability to produce a detectable amount of A factor (less than 0.5% of that produced by parental strain 13189) and thus failed to produce both streptomycin and aerial mycelia. Both processes, however, could be restored by adding 1  $\mu\text{g}$  of A factor per ml exogenously. This mutant defective in A-factor synthesis (*afs*) may be classified as an *afsA* or *afsB* mutant (or as an *afsA afsB* mutant deficient in both genotypes), as described by Hara et al. (13). The ability to form aerial mycelia could not be restored by the addition of decoyinine in any experimental conditions tested.

Deficiency in streptomycin production by the *rel* and *afs* mutants could result from a failure of induction of enzymes involved in streptomycin biosynthesis. Two enzymes, amidinotransferase and streptomycin kinase, were used as probes. Their activities were coordinately expressed just before the beginning of streptomycin production, but neither the *rel* mutant nor the *afs* mutant was capable of inducing these enzymes (Table 1). As examined in vitro, the physiological concentrations of ppGpp (0.2 to 3 mM) or A factor (0.1 to 5  $\mu\text{g}/\text{ml}$ ) did not affect the amidinotransferase activity (not examined for streptomycin kinase). Thus, the failure of

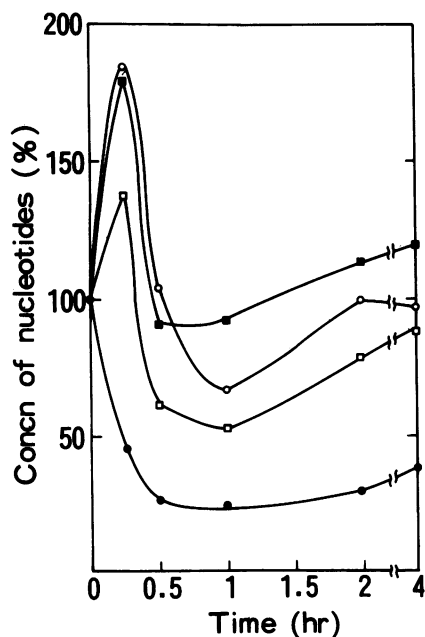


FIG. 2. Changes in the intracellular concentrations of nucleoside triphosphates after the addition of decoyinine. Cells of strain 13189 were grown in SPY medium for 6 h. Decoyinine was then added to each flask to give a final concentration of 0.3 mM, and incubation was further continued. At the indicated times, cells in each flask were quickly filtered and extracted, and the extracts were analyzed by HPLC. The nucleotide contents at zero time (picomoles per milligram [dry weight]) were as follows: GTP, 1670; ATP, 2440; UTP, 1210; and CTP, 460. Symbols: ●, GTP; ○, ATP; □, UTP; ■, CTP.

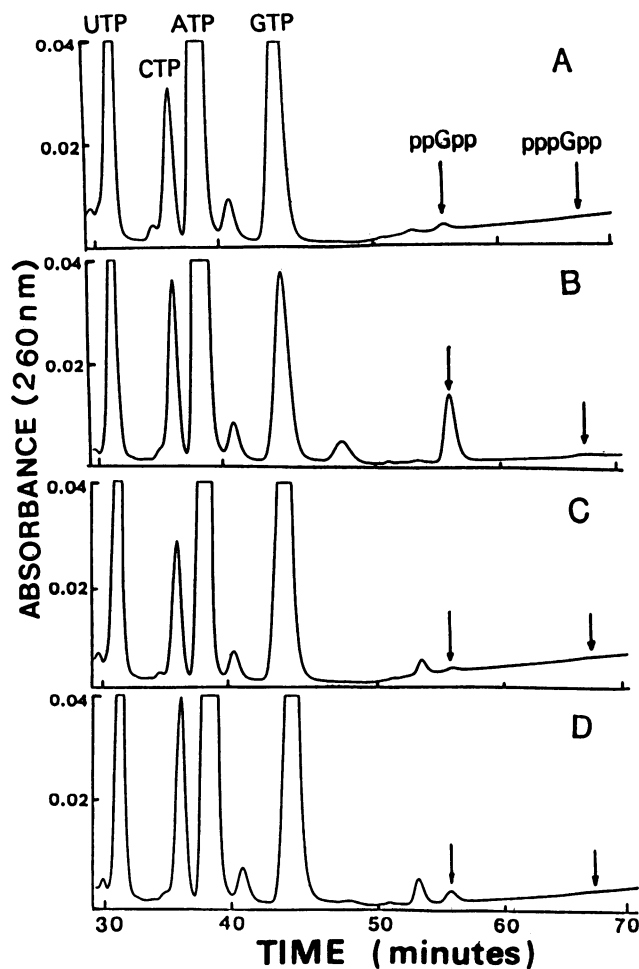


FIG. 3. Elution profiles of nucleotides extracted from cells of the parental (13189) or mutant (3-3) strain after deprivation of Casamino Acids. The strains, grown to mid-exponential phase (10 h for the parent strain and 12 h for the mutant) in synthetic medium plus 1% Casamino Acids, were harvested and transferred to fresh synthetic medium (without Casamino Acids). After 10 min of incubation with shaking, cells were collected and extracted and the extracts were analyzed by HPLC. Samples applied were equivalent to 6.1 to 6.8 mg of cells (dry wt) in each profile. (A) Parent, zero time. (B) Parent, 10 min. (C) Mutant 3-3, zero time. (D) Mutant 3-3, 10 min.

enzyme induction was a cause for the deficiency in streptomycin production. The failure to induce streptomycin kinase accounted for the decreased resistance of the *rel* mutant to streptomycin (see above), since this enzyme is also involved in the inactivation of streptomycin present intracellularly.

The *afs* mutant loses the capability of producing NADP-glycohydrolase (12, 37). In contrast, the *rel* mutant produced it normally (Table 1).

**Changes in nucleotide pool after nutritional shift down.** I investigated how *S. griseus* exerts the stringent response, i.e., accumulation of ppGpp or pppGpp, by approaches similar to those used with *B. subtilis* and *Streptomyces* sp. MA406-A-1 (20, 24). The strains were grown to mid-exponential phase in synthetic medium supplemented with 1% Casamino Acids; cells were harvested by filtration and immediately resuspended in fresh synthetic medium lacking Casamino Acids. Cells of the *rel*<sup>+</sup> parental strain accumulated ppGpp immediately after shift down (Fig. 5). The other

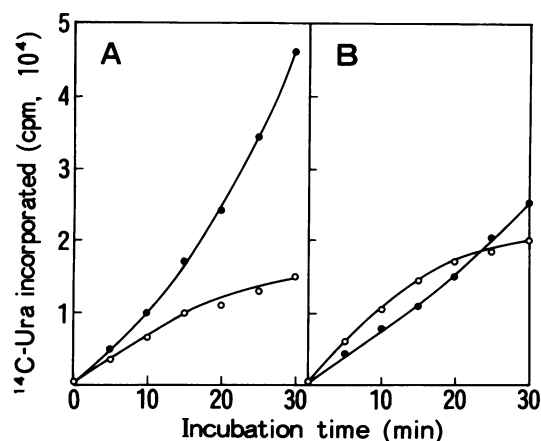


FIG. 4. RNA synthesis by cells of the parental strain (13189) and a mutant (3-3) after deprivation of Casamino Acids. Cells, grown with 1% Casamino Acids as described in the legend to Fig. 3, were collected and transferred to give an optical density at 600 nm of 2 in synthetic medium containing [2-<sup>14</sup>C]uracil with (●) or without (○) Casamino Acids and incubated with shaking for 30 min. (A) Parental strain. (B) Mutant 3-3.

nucleotides assayed (ATP, UTP, and CTP) showed a transient 1.2- to 1.9-fold increase, maximal at 15 min, and then decreased to the initial level 4 h after shift down (data not shown). The *afs* mutant 14 also accumulated as much ppGpp as the parental strain did. The result indicates that A factor was unnecessary for the accumulation of ppGpp. The *rel* mutant 3-3 accumulated ppGpp only to about 15% of that accumulated by the parental strain (Fig. 5). The accumulation of pppGpp (not shown) was far less than that of ppGpp; it was at most 60 pmol/mg (dry weight) in the parent and *afs* mutant and 10 pmol in the *rel* mutant. The accumulation of ppGpp and pppGpp during deprivation of Casamino Acids was inhibited by 70 to 90% by chloramphenicol (4 µg/ml), with virtually no effect on the growth rate of cells. If magnesium sulfate present in the medium at a high concentration was reduced to 0.1%, the GTP pool of the *rel*<sup>+</sup> parental strain decreased markedly after shift down, but in the *rel* mutant, the decrease was much smaller (Table 2), as was also reported for *B. subtilis* (20, 26).

The parental strain, but not the *rel* mutant, began producing streptomycin 1 to 2 h after shift down.

**Sporulation in submerged culture.** *S. griseus* has the characteristic of forming spores (submerged spores) directly from vegetative mycelia if cultured in suitable liquid medium. This morphological differentiation was also investi-

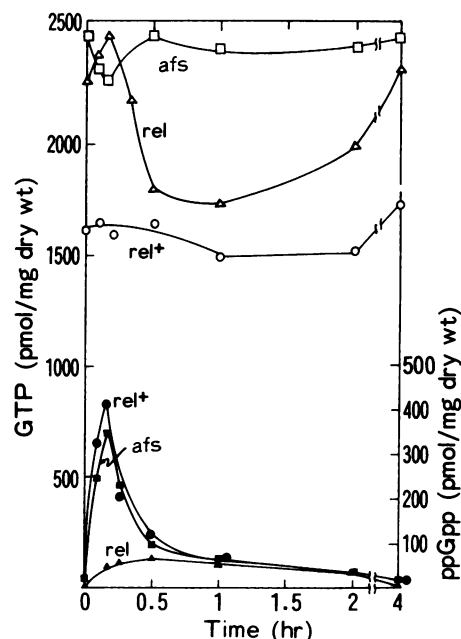


FIG. 5. Changes in the intracellular concentrations of ppGpp and GTP after shift down from synthetic medium containing 1% Casamino Acids to synthetic medium lacking Casamino Acids. Cells of the parental strain (13189, *rel*<sup>+</sup>) or mutants (3-3, *rel*; 14, *afs*), grown to mid-exponential phase in synthetic medium plus 1% Casamino Acids as described in the legend to Fig. 3, were harvested and transferred to fresh synthetic medium without Casamino Acids and then incubated at 30°C with shaking. At the indicated times, cells were filtered and extracted and the nucleotides were quantified by HPLC. Closed symbols, ppGpp; open symbols, GTP.

gated with respect to its initiation process, comparing it with that of aerial mycelium formation.

Parental strain 13189 produced submerged spores abundantly after 48 h of cultivation in sporulation medium (Table 3). These spores were refractile when observed under a phase-contrast microscope and highly resistant to sonication, as reported by Kendrick and Ensign (16). If the sporulation medium was supplemented with 1% Casamino Acids, both the growth rate and final biomass were markedly enhanced, whereas spore titers decreased by a factor of 4. The addition of 1 mM decoyinine at mid-exponential growth, however, shortened the time of appearance of spores and the cells eventually produced abundant spores. These results are consistent with what was observed in the development of aerial mycelia (Fig. 1). The *rel* mutant had a reduced ability to sporulate but produced spore titers almost as high as those

TABLE 1. Changes in specific activity of amidinotransferase, streptomycin kinase, and NADP-glycohydrolase in parental and mutant strains

Strain	Sp act (U/mg of protein) <sup>a</sup> at various times (h)									
	Amidinotransferase <sup>b</sup>				Streptomycin kinase <sup>b</sup>				NADP-glycohydrolase <sup>c</sup>	
	8	24	48	72	8	24	48	72	8	24
Parent ( <i>rel</i> <sup>+</sup> <i>afs</i> <sup>+</sup> )	0.001	0.055	0.071	0.010	0.01	1.04	0.96	1.00	3,390	3,150
Mutants										
3-3 ( <i>rel</i> )	0.002	0.002	0.001	0.001	0.05	0.04	0.03	0.08	2,880	7,930
14 ( <i>afs</i> )	0.002	0.003	0.001	0.002	0.02	0.01	0.02	0.02	<50	<50

<sup>a</sup> For definitions of units, see Materials and Methods.

<sup>b</sup> Strains were grown in SPY medium.

<sup>c</sup> Strains were grown in GYM medium.



TABLE 2. Changes in the intracellular concentrations of nucleotides after Casamino Acids deprivation<sup>a</sup>

Strain	Time (min)	Nucleotide concn (pmol/mg [dry wt])			
		ATP	GTP	ppGpp	pppGpp
Parent ( <i>rel</i> <sup>+</sup> )	0	13,600	4,610	22	<8
	10	17,600	3,090	476	51
	30	19,300	2,260	220	9
3-3 ( <i>rel</i> )	0	9,370	4,700	19	<8
	10	15,600	4,710	105	8
	30	18,300	4,090	98	<8

<sup>a</sup> Experimental conditions were the same as those described in the legend to Fig. 5, except that the medium contained 0.1% instead of 1% magnesium sulfate before and after transfer.

produced by the *rel*<sup>+</sup> parental strain with added decoyinine. The *afs* mutant failed to produce spores. This deficiency was restored completely by adding A factor. As observed in SPY medium (Fig. 2), the GTP pool size in these strains decreased by 50 to 70% 1 h after decoyinine addition, without an increase in ppGpp. When both A factor and decoyinine were added to the *afs* mutant, the mutant produced abundant spores at an early time; this was especially pronounced in sporulation medium supplemented with Casamino Acids, in which a spore titer of  $6 \times 10^8$ /ml was counted even at 30 h (Table 3).

The changes in the nucleotide pools of each strain growing in sporulation medium were examined (Fig. 6). The pool size of ppGpp in the parental strain was very low during mid-exponential growth (8 h after inoculation), but it increased to 82 pmol/mg (dry weight) during post-exponential growth (15 h after inoculation). The pool size of GTP decreased concomitantly with the increase in ppGpp. This sharp decrease in GTP content could be a metabolic trigger for the development of submerged spores. The *rel* mutant accumulated smaller amounts of ppGpp, and the decrease in GTP content was less pronounced, accounting for the reduced ability to sporulate. The *afs* mutant accumulated as much ppGpp as the parental strain did, but unexpectedly, the decrease in GTP was less than that observed in the parental strain. The reduced decrease in GTP was not repaired by the addition of A factor (Fig. 6).

## DISCUSSION

The purpose of this study was to stress the significance of the stringent response and GTP content in the differentiation of streptomycetes and to clarify the role of GTP and the stringent response in comparison with that of A factor. The results indicate that in *S. griseus* ppGpp may be an early signal, produced in response to nutrient limitation, which may trigger events such as streptomycin production. This suggestion is supported by the behavior of the *rel* mutant and by the response of the parental strain to the nutritional condition of the medium. The *rel* mutations also abolished

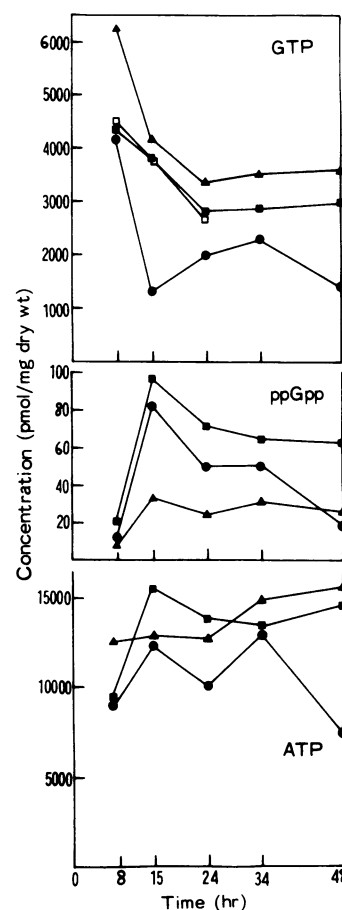


FIG. 6. Changes in the intracellular concentrations of ppGpp, GTP, and ATP during growth in sporulation medium. Spores of the parental strain (●), the *rel* mutant (▲), or the *afs* mutant (■) were inoculated in sporulation medium and incubated at 30°C as described in Materials and Methods. The *afs* mutant was also cultivated in sporulation medium supplemented with 0.5 μg of A factor per ml (added just after inoculation). □, GTP concentrations.

formycin and actinomycin production by *Streptomyces* spp. (24, 25). As relaxed (*relA* and *relC*) mutants of *B. subtilis* also fail to produce an antibiotic (27), ppGpp may be a general regulatory molecule for triggering secondary metabolism in procaryotes. This is an intriguing notion in light of the fact that ppGpp has been detected among extremely widespread (if not all) procaryotes (see references 1, 10, 22, 30, 32, and 35 for streptomycetes). I cannot rule out completely the possibility that the changes in ribosomal conformation per se resulting from *rel* mutation, rather than from ppGpp, were responsible for the inability to produce antibi-

TABLE 3. Submerged spore formation by parental and mutant strains

Addition(s) to sporulation medium	No. of submerged spores/ml at various times (h)							
	Parent ( <i>rel</i> <sup>+</sup> <i>afs</i> <sup>+</sup> )		3-3 ( <i>rel</i> )		14 ( <i>afs</i> )		14 + A factor <sup>a</sup>	
	30	48	30	48	30	48	30	48
None	$2 \times 10^5$	$3 \times 10^8$	$<2 \times 10^5$	$4 \times 10^7$	$<2 \times 10^5$	$<2 \times 10^5$	$3 \times 10^5$	$2 \times 10^8$
1% Casamino Acids	$<2 \times 10^5$	$8 \times 10^7$	$<2 \times 10^5$	$4 \times 10^7$	$<2 \times 10^5$	$3 \times 10^5$	$8 \times 10^7$	$1.5 \times 10^9$
1% Casamino Acids + decoyinine <sup>b</sup>	$3 \times 10^6$	$5 \times 10^8$	$2 \times 10^6$	$2 \times 10^8$	$2 \times 10^6$	$5 \times 10^7$	$6 \times 10^8$	$2.5 \times 10^9$

<sup>a</sup> A factor (final concentration, 0.5 μg/ml) was added just after inoculation.

<sup>b</sup> Decoyinine (final concentration, 1 mM) was added at mid-exponential growth (8 h for the parental strain and strain 14 or 12 h for strain 3-3 after inoculation).

otics. This possibility is, however, unlikely, because a *rel* mutant of *Streptomyces* sp. MA406-A-1 regains the capability of producing formycin, accumulating ppGpp under glucose deprivation (24). Since streptomycin production started soon after Casamino Acids deprivation, it is especially of interest whether the effect of ppGpp on the expression of such secondary genes coding amidinotransferase or streptomycin kinase is elicited by the direct function of ppGpp on RNA polymerase, which might in turn change the transcriptional selectivity dramatically.

In *E. coli*, ppGpp inhibits IMP dehydrogenase, which plays a role in GMP (and thus GTP) synthesis (8). A recent enzymatic study, which will be reported elsewhere, also showed that ppGpp is an outstandingly strong inhibitor of *S. griseus* IMP dehydrogenase among the normal nucleotides tested. Thus, the elevated levels of ppGpp should be responsible for the sharp decrease in GTP pools of the parental strain (Table 2 and Fig. 6). The stringent response, therefore, would play a role, although indirectly, in the initiation of submerged spore formation and aerial mycelium formation by *S. griseus*. This notion is consistent with the fact that the appearance of aerial mycelia is always delayed drastically in the *rel* mutants of *Streptomyces* spp. (24, 25, and this study). If the stringent response was impeded (e.g., by a *rel* mutation), the decrease in the GTP pool might depend almost solely on the exhaustion of nutrients, which might result in a curtailment of purine nucleotide synthesis. This accounts for the increased sensitivity of the *rel* mutants to nutrients in the development of aerial mycelia. Because the method developed by Kendrick and Ensign (16) for inducing sporulation of *S. griseus* depends on a deprivation of nitrogen or phosphate source, it is highly likely that the stringent response or the curtailment of de novo purine synthesis (or both) leading to abrupt GTP decrease was responsible for the timely onset of sporulation.

The differences in action of the *rel* mutant and A-factor response are especially intriguing. Although both the *rel* gene function and A factor were essential for streptomycin production and for abundant spore formation, it is evident that the synthetic processes of ppGpp and A factor proceeded independently, eliminating the particular dependent mechanisms, A factor → *rel* (ppGpp) → enzyme synthesis, or *rel* (ppGpp) → A factor → enzyme synthesis. Strikingly, the decrease in the GTP pool was less pronounced in the *afs* mutant and was not repaired by the addition of A factor (Fig. 6). The mechanisms involved in this phenomenon remain unknown, but it may imply widespread pleiotropic effects of the gene(s) determining A-factor synthesis of *S. griseus*. Since A factor alone could not initiate aerial mycelium formation in the presence of excess nutrients, it may have a role in making the cells competent to adapt to environmental changes, as suggested earlier by Gräfe et al. (9). The remarkable synergistic effect produced by the combination of decoyinine and A factor (Table 3) supports their suggestion.

#### ACKNOWLEDGMENTS

I thank J. E. Grady and T. Beppu for their donation of drugs and M. Okuhara and M. Kohsaka for their encouragement to proceed with this study.

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